Drug-Susceptibility and Isolation of a Plasmid in *Haemophilus (Actinobacillus) pleuropneumoniae*

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ABSTRACT. We isolated 56 *Haemophilus (Actinobacillus) pleuropneumoniae* strains from the pneumonic porcine lung tissues and tested them for antimicrobial susceptibility. Two drug-resistant strains were obtained. One, named KH-265, was resistant to streptomycin (SM) and sulfonamide (SA), and the other, named KH-195, was resistant to tetracycline (TC). The minimum inhibitory concentrations (MICs) of drugs for resistant strains were 100 µg/ml for SM, 3200 µg/ml for SA, and 12.5 µg/ml for TC. KH-265 possessed a 8.3Kb nonconjugative plasmid, pMS260, encoding SM and SA resistance, which was transformable to *E. coli* strains. pMS260 belonged to none of 14 incompatibility groups including Inc. P and Inc. Q, so far tested. It was mobilizable to various causative strains for respiratory infections, *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Pasteurella multocida* and *Haemophilus pleuropneumoniae*, by RP₄ (Inc. P) plasmid.—**KEY WORDS:** drug susceptibility, *Haemophilus (Actinobacillus) pleuropneumoniae*, resistance plasmid.

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Haemophilus (Actinobacillus) pleuropneumoniae is a hemolytic gram-negative bacteria requiring β-nicotinamide adenine dinucleotide (β-NAD) for growth and is a respiratory pathogen responsible for fibrinous, hemorrhagic, necrotizing pleuropneumonia of swine [6, 13, 14, 17, 27, 29]. It can affect pigs of any age, and is especially common in young and rapidly growing swine [6, 7, 25, 30, 34]. In addition to pneumonia, it can also cause verrucous endocarditis and septicemia [9, 33].

Since the disease was first identified by Pattison *et al.* in 1957 [24], many studies have been reported, but there is only one report about R plasmid of *H. pleuropneumoniae* [6]. This report describes the drug susceptibility of *H. pleuropneumoniae* from pneumonic porcine lung and a new nonconjugative plasmid of wide host range.

MATERIALS AND METHODS

Bacterial strains: Nalidixic acid resistant (NA^r) or rifampicin resistant (RFP^r) *E. coli* K-12 χ1037 (*hsdR*) strains [10], *Pseudomonas aeruginosa* PAO2142-RFP^r, *Bordetella bronchiseptica* ATCC4617-RFP^r, *Pasteurella multocida* HI401-RFP^r, *Branhamella catarrhalis* TY-105 [37], *Haemophilus influenzae* ML6146 and *H. pleuropneumoniae* HI181-RFP^r were used as recipients of plasmid. *B. bronchiseptica* ATCC4617-RFP^r and *H. influenzae* ML6146 were kindly supplied by Dr. M. Shimizu, Department of Hygiene, Zen-noh Central Institute for Feed and Livestock Research and Mr. S. Oouchi, Maebashi Red Cross Hospital. The plasmids used were all from the collection in our laboratory.

Media and drugs: As liquid media, BHI (brain heart infusion)-broth (Nissui, Tokyo, Japan) and L-broth [16] were used routine-

ly, and BHI and L-broth supplemented with 25 $\mu\text{g/ml}$ β -NAD (Sigma, Missouri, USA) (V-BHI) (V-L) were used for *H. pleuropneumoniae* strains. For *H. influenzae* strains, BHI supplemented in 2 $\mu\text{g/ml}$ β -NAD and 10 $\mu\text{g/ml}$ Hemin (Sigma) (XV-BHI) was used. For *P. aeruginosa* strains, L-broth supplemented with 0.4% KNO_3 was used.

As solid media, Muller Hinton agar (MHA) (OXOID, Hants, England) and Tryptso-soya agar (TSA) (Nissui) and BTB-lactose agar (BTB) (Eiken, Tokyo, Japan) were used routinely, and MHA and TSA supplemented with 25 $\mu\text{g/ml}$ β -NAD (V-MHA) (V-TSA) were used for *H. pleuropneumoniae* strains. For *H. influenzae* strains, MHA and TSA supplemented with 2 $\mu\text{g/ml}$ β -NAD and 10 $\mu\text{g/ml}$ Hemin (XV-MHA) (XV-TSA) were used. For *P. multocida* and *B. catarrhalis* strains, MHA and TSA were used.

The drugs used and the abbreviations: sulfamethoxazol (SA); trimethoprim (TMP); nalidixic acid (NA); penicillin G (PCG); ampicillin (ABPC); cefaloridine (CER); streptomycin (SM); kanamycin (KM); tetracycline (TC); chloramphenicol (CP); erythromycin (EM); tylosin (TS); lincomycin (LCM); colistin (CL); rifampicin (RFP); mercuric chloride (HG); spectinomycin (SPCM).

Antibiotic susceptibility test: Minimum inhibitory concentrations (MICs) were determined by agar dilution method standardized by the Japan Society of Chemotherapy [22].

Isolation of plasmid DNA: The method of Inoue *et al.* [8] was used for small scale preparation of DNA. For large scale preparation, plasmid DNA was isolated by ethidium bromide CsCl equilibrium centrifugation of the cleared lysate obtained by treatment of cells with Triton-X as described by Kuperzstoch-Portnoy *et al.* [15].

Transformation: *E. coli* K-12 $\chi 1037\text{NA}^r$ (*hdsR*) cells treated with calcium chloride

were transformed with plasmid DNA as described by Curtis *et al.* [4]. As a reference plasmid, Rlb679 plasmid encoding resistance to both SM and SA, and belonging to the Inc.Q group [3] was used.

Restriction endonuclease treatment of plasmid DNA: Plasmid DNA preparations were digested at 37°C for 2 hr with restriction endonucleases, *EcoRI*, *AvaI* and *PstI*. The digested DNA was then subjected to electrophoresis with a 0.8% horizontal agarose gel at 30mA for 18 hr.

Incompatibility testing: Incompatibility testing was done as previously described by Sagai *et al.* [26]. The standard plasmids of known incompatibility groups were introduced by conjugation or by transformation into *E. coli* K12 $\chi 1037\text{NA}^r$ containing the *H. pleuropneumoniae* plasmid. The non-transmissible plasmid of *H. pleuropneumoniae* origin was introduced by transformation to the strains bearing standard plasmids. Selection was made for incoming plasmids in each case. Ten to thirty of the colonies that grew on the selection plates were purified three times, using the same medium, and confirmed if they had drug resistance markers conferred by both plasmids.

Mating and mobilization: The donor and the recipient strains at the logarithmic phase of growth were mixed at a ratio of 1:4 and incubated with gently shaking at 37°C for 15 min. An aliquot of the mixture (0.05 ml) was spread on BTB or MHA plates containing selective drugs for transconjugants.

From *E. coli* to various species of strains, RP₄ (Inc. P) and R446b (Inc. M) were used as mobilizers. The donor (0.1 ml) and the recipient (0.1 ml) cultures at the logarithmic phase were mixed with supplement of fresh broth (0.8 ml) and incubated without shaking at 37°C for overnight. When *B. catarrhalis* was used as a recipient, however, membrane filter method was used. An aliquot of the mixture (0.05 ml) was spread on the agar

plates containing selective drugs for trans-conjugants. Different agar plates were used, depending on the recipient strains, those were, V-TSA and V-MH for *H. pleuropneumoniae*, XV-TSA and XV-MH for *H. influenzae*, TSA, MH and BTB for other strains.

RESULTS

Bacterial isolates: During 1987 and 1988, we isolated 56 *H. pleuropneumoniae* strains from the lungs of 330 slaughtered pigs, which had been raised at 56 farms in different geographic districts of Gunma and Niigata prefecture. We cultured porcine lung tissues on TSA plates supplemented with 5% horse blood and 25 µg/ml β-NAD, and then incubated the plates at 37°C for 18–24 hours. The *H. pleuropneumoniae* isolates were confirmed for their serotype by the RPA test [19], one of them, KH-195 was identified as serovar 5 and the remaining 55 strains were identified as serovar 2.

Antibiotic susceptibility test: The MICs of antimicrobial drugs for 56 *H. pleuropneumoniae* strains are shown in Table 1. TMP

and β-lactam antibiotics, PCG, ABPC, were highly active (MIC; < 0.1 to 0.4 µg/ml), and CER showed lower activity (MIC; 0.4 to 1.6 µg/ml). NA, TC, CP, CL, REP and HG were moderately active (MIC; 0.2 to 12.5 µg/ml).

Of 56 isolates, two strains were resistant to one or two drugs, that is, a strain KH-195 was resistant to TC, and another strain KH-265 was resistant to both SM and SA.

Isolation of drug-resistance plasmid: None of drug-resistance was transferred by conjugation from either KH-265 or KH-195. We could detect plasmid DNA from KH-265 but not from KH-195. *E. coli* K-12 strain χ1037-NA^r was transformed with plasmid DNA from KH-265. With small scale DNA preparation, approximately 4×10⁴ SM^r transformants were obtained using 1 µg of DNA, when selected by BTB agar supplemented with SM. All SM^r colonies examined (30 colonies) were SA-resistant, indicating that the plasmid mediated resistance to both SM and SA. We designated the plasmid pMS260.

MICs were determined for the transformants and compared with that of the strain

Table 1. Antibiotic susceptibility of *H. pleuropneumoniae* strains^{a)}

Drug	Minimum inhibitory concentration (μg/ml)																
	≤0.1	0.2	0.4	0.8	1.6	3.1	6.3	12.5	25	50	100	200	400	800	1600	3200	6400
SA								1	30	15	7	2				1	
TMP	56																
NA			3	42	11												
PCG	1	26	29														
ABPC	13	43															
CER			1	38	17												
SM						8	33	14			1						
KM					13	30	13										
TC			19	35	1			1									
CP			37	19													
EM					9	16	31										
TS								5	33	18							
LCM							6	44	6								
CL		1	11	44													
RFP		7	23	26													
HG				8	48												

a) Fifty-six strains were examined.

bearing Rlb679 (SM^rSA^r) (Table 2). Both plasmids, pMS260 and Rlb679 were resistant to SM and SA, but sensitive to SPCM.

Restriction endonuclease analysis of plasmid DNA: Both pMS260 and the reference plasmid Rlb679 were digested with endonucleases and the number and the size of fragments obtained were estimated by agarose gel electrophoresis and compared each other. As shown in Table 3, both plasmids

were not the same.

Incompatibility testing: The results of incompatibility testing are shown in Table 4. pMS260 could coexist with any of 14 plasmids so far tested.

In the combinations of pMS260 with R446b (Inc. M), RP₄ (Inc. P), or R6K (Inc. X), we obtained transconjugants that accepted only pMS260 after mobilized transfer from the donor carrying both plasmids (Table 5). In the combination of pMS260 with each of other plasmids, the conjugative plasmids transferred to *E. coli* strains without mobilization of pMS260, and we isolated pMS260 DNA from each strain carrying both pMS260 and either one of these 11 conjugative plasmids by small scale

Table 2. MICs of drugs for *Haemophilus pleuropneumoniae* and *Escherichia coli*

Strain	MIC(μ g/ml)		
	SM	SA	SPCM
KH-5 ^{a)}	6.3	25	25
KH-265 ^{b)}	100	3200	25
χ 1037-NA ^r ^{c)}	1.6	50	25
χ 1037-NA ^r (pMS260) ^{d)}	400	6400	25
χ 1037-NA ^r (Rlb679) ^{d)}	100	6400	25

a) An antibiotic susceptible strain of *H. pleuropneumoniae*.

b) *H. pleuropneumoniae* bearing pMS260.

c) A derivative strain of *E. coli* K-12.

d) Transformant.

Table 3. Number of pMS260 DNA fragment(s) obtained by digestion with restriction endonucleases

	<i>Pst</i> I	<i>Ava</i> I	<i>Eco</i> RI	Molecular size (Kb)
Rlb679	2	5	1	8.9
pMS260	2	3	2	8.3

Table 4. Incompatibility test for pMS260(A)

Plasmid in donor	Resistance marker	(Inc.)	Selection	No. of transconjugants bearing both plasmids	
				No. of transconjugants examined (Ratio)	
R386	TC	(F I)	TC	(10/10)	
R9-5	CM	(F II)	CM	(10/10)	
R124	TC	(F IV)	TC	(10/10)	
R621a	TC	(I γ)	TC	(30/30)	
R391	KM, HG	(J)	KM	(10/10)	
R387	CM, SM	(K)	CM	(10/10)	
R446b	TC, SM	(M)	TC	(10/10)	
RP ₄	ABPC, KM, TC	(P)	KM	(10/10)	
Rlb679::Tn5	SA, KM	(Q)	KM	(30/30)	
pMS21::Tn3	SA, ABPC	(Q)	ABPC	(10/10)	
Rts-1	KM	(T)	KM	(10/10)	
R388	SA, TMP	(W)	TMP	(10/10)	
R6K	ABPC	(X)	ABPC	(10/10)	
pBR322	ABPC, TC		ABPC	(10/10)	
pSC101::Tn5	TC, KM		KM	(30/30)	
pMS260::Tn5	SA, KM		KM	(0/30)	

Donor strain, χ 1037-PFP^r. Recipient strain, χ 1037-NA^r carrying pMS260. Rlb679::Tn5, pMS21::Tn3, pBR322 and pSC101::Tn5 were introduced by transformation and the other plasmids were by conjugation.

preparation method. When pMS260 coexisted with Rlb679::Tn5, pMS21::Tn3 (Inc. Q), pBR322 or pSC101::Tn5, we isolated each plasmid independently.

It was concluded that no recombinant

pMS260 with another plasmid was detected in any combinations, because we identified pMS260 separately from another plasmid coexisting in transconjugants. When incompatibility testing was performed between pMS260 and pMS260::Tn5 (SM^s, SA^r, KM^r), they could not coexist in the same cell. The results indicated that pMS260 did not belong to any of 14 incompatibility groups tested.

Transfer of pMS260 by mobilization:
From *E. coli* to *E. coli*: As shown in Table 5, pMS260 was mobilized to *E. coli* strain by RP₄ (Inc. P), R446b (Inc. M) and R6K (Inc. X) among 11 conjugative plasmids. By R6K, pMS260 was transferred at a 200 times lower frequency than the mobilizer R6K. It was transferred at the same frequency as the mobilizer RP₄. By the mobilizer R446b, pMS260 was transferred at a more than 50 times higher frequency than that of R446b, although Rlb679 transferred at the same frequency as R446b (data not shown).

From *E. coli* to various species of strains: As shown in Table 6, pMS260 and Rlb679 were transferred by mobilization with RP₄ to *E. coli*, *P. aeruginosa* and *B. bronchiseptica* at almost the same frequency as RP₄. To *P. multocida* and *H. pleuropneumoniae*, pMS260 was transferred, although the

Table 4. Incompatibility test for pMS260 (B)

Plasmid in recipient	(Inc.)	No. of transformants bearing both plasmids / No. of transformants examined (Ratio)
R386	(F I)	(10/10)
R9-5	(F II)	(10/10)
R124	(F IV)	(10/10)
R621a	(I γ)	(29/30)
R391	(J)	(10/10)
R387	(K)	(10/10)
R446b	(M)	(10/10)
RP ₄	(P)	(10/10)
Rlb679::Tn5	(Q)	(29/30)
pMS21::Tn3	(Q)	(10/10)
Rts-1	(T)	(10/10)
R388	(W)	(10/10)
R6K	(X)	(10/10)
pBR322		(10/10)
pSC101::Tn5		(27/30) ^{a)}
pMS260::Tn5		(0/30)

Donor strain, χ 1037-NA^r carrying pMS260. Recipient strain, χ 1037-RFP^r. Plasmid pMS260 was introduced by transformation, and the SM or SA resistance was used for the selection marker.

a) Control experiment showed that pSC101::Tn5 in recipient was spontaneously lost at the similar frequency from the cells without infection of pMS260.

Table 5. Mobilization of pMS260 by plasmids R446b, RP₄ or R6K

Plasmids in donor	Inc. of mobilizer plasmid	Selective drug	Transfer frequency ^{a)}	No. of transconjugants carrying			Mobilization frequency ^{b)}
				pMS260	mobilizer plasmid	both	
pMS260/R446b	(M)	SA	3×10^{-4}	20/20	0	0	5×10^1
		TC	6×10^{-6}	0	11/20	9/20	
pMS260/RP ₄	(P)	SM	4×10^{-4}	18/20	0	2/20	1×10^0
		CBPC	3×10^{-4}	0	7/20	13/20	
pMS260/R6K	(X)	SM	2×10^{-6}	16/20	0	4/20	5×10^{-3}
		ABPC	4×10^{-4}	0	20/20	0	

Donor strain, χ 1037-RFP^r. Recipient strain, χ 1037-NA^r.

a) After 15 minutes incubation.

b) Mobilization frequency was expressed as the ratio of transfer frequency of pMS260 to that of mobilizer plasmid.

Table 6. Mobilization of pMS260 or Rlb679 by PP₄ to recipients of various species

Recipient	Selective drug	pMS260				Rlb679			
		Transfer frequency	No. of transconjugants carrying			Transfer frequency	No. of transconjugants carrying		
			pMS260	RP ₄	both		Rlb679	RP ₄	both
<i>E. coli</i>	SM	3×10^0	0	0	30/30	4×10^0	0	0	30/30
χ 1037-RFP ^r	CBPC	6×10^0	0	12/30	18/30	4×10^0	0	3/30	27/30
<i>P. aeruginosa</i>	SM	4×10^{-5}	24/30	0	6/30	7×10^{-4}	27/30	0	3/30
PAO2142-RFP ^r	CBPC	1×10^{-5}	0	6/30	24/30	7×10^{-4}	0	4/30	26/30
<i>P. multocida</i>	SM	4×10^{-4}	30/30	0	0	$< 3 \times 10^{-9}$			
HI401-RFP ^r	KM	$< 2 \times 10^{-9}$				$< 3 \times 10^{-9}$			
<i>B. bronchiseptica</i>	SM	6×10^{-1}	0	0	30/30	3×10^0	0	0	30/30
ATCC4617-RFP ^r	KM	2×10^0	0	23/30	7/30	4×10^0	0	12/30	18/30
<i>B. catarrhalis</i>	SM	$< 2 \times 10^{-9}$				$< 2 \times 10^{-9}$			
TY-105	KM, ABPC or TC	$< 2 \times 10^{-9}$				$< 2 \times 10^{-9}$			
<i>H. influenzae</i>	SM	$< 4 \times 10^{-9}$				$< 3 \times 10^{-9}$			
ML6146	KM, ABPC or TC	$< 4 \times 10^{-9}$				$< 3 \times 10^{-9}$			
<i>H. pleuropneumoniae</i>	SM	3×10^{-4}	30/30	0	0	$< 3 \times 10^{-9}$			
HI181-RFP ^r	KM, ABPC or TC	$< 4 \times 10^{-9}$				$< 3 \times 10^{-9}$			

Table 7. MICs for various strains bearing pMS260^{a)}

Strain	MIC(μ g/ml)	
	SM	SA
PAO2142-RFP ^r	3.2	400
PAO2142-RFP ^r (pMS260)	>800	>6400
HI401-RFP ^r	12.5	100
HI401-RFP ^r (pMS260)	800	1600
ATACC4617-RFP ^r	100	1.6
ATACC4617-RFP ^r (pMS260)	>800	6400
HI181-RFP ^r	6.3	25
HI181-RFP ^r (pMS260)	100	3200

a) The causative bacteria for infectious disease of the respiratory organs were examined.

mobilizer RP₄ did not transfer to them. While Rlb679 was not transmissible to those strains.

When R446b was used as the mobilizer, Rlb679 was transferred to *E. coli* strain at almost the same frequency as R446b. While pMS260 was transferred at a 20 times lower frequency than R446b. They were not transferred to any of the other strains (data not shown).

MICs for various strains bearing pMS260

were determined (Table 7). In all strains, MICs of SM and SA were raised by acquisition of pMS260.

DISCUSSION

The in vitro susceptibility patterns of *H. pleuropneumoniae* to 16 drugs in the present paper were similar to other reports about *H. pleuropneumoniae* by Inoue *et al.* [7], Gilbride *et al.* [5] and Shimizu *et al.* [32], and also similar to the results from other *Haemophilus* species, *H. influenzae* [20] and a causative agent of pneumonia, *Pasteurella multocida* [32].

We isolated two resistant strains among 56 isolates. One of them, KH-195 was singly resistant to TC, and belonged to serovar 5. The other strain, KH-265 was doubly resistant to SM and SA and belonged to serovar 2. Nicolet *et al.* [21] described that 98 *H. pleuropneumoniae* strains belonging to serovar 2 were sensitive to 12 drugs tested. Inoue *et al.* [7] also described 105 *H. pleuropneumoniae* strains belonging to serovar 2 were sensitive but each one of serovar

3 and 5 *H. pleuropneumoniae* strains were resistant to drugs. Libal *et al.* [17], Sebunya *et al.* [28], and Schultz *et al.* [30] found many strains resistant to several drugs by a disk diffusion technique, but the serotypes were not determined. Kawahara *et al.* [12] and Suzuki *et al.* [36] have reported that *H. pleuropneumoniae* strains including serovar 2 were resistant to several drugs. These results suggest that drug resistant strains of *H. pleuropneumoniae* are increasing in recent years in Japan.

It is not surprising that such resistant *H. pleuropneumoniae* have emerged, because antibiotics have been used commercially in animal feeds for improving weight gain and feed efficiency. This intense selective pressure by antibiotics on farms have led to a widespread increase in R plasmid containing bacteria [35]. Among bacteria isolated from domestic animals, extensive prevalence of R plasmids has been demonstrated in *E. coli* [11, 18] and *B. bronchiseptica* [31, 38].

The genes responsible for resistance to SM and SA in *H. pleuropneumoniae* KH-265 were located on a plasmid, pMS260 (8.3 Kb in size). This plasmid did not possess the genes necessary for conjugal transmission to *H. pleuropneumoniae* or to *E. coli*. The SM resistance mediated by the plasmid appeared to be based on SM-phosphotransferase, because pMS260 did not encode resistance to SPCM [23]. Hirsh *et al.* [6] isolated two plasmids from *H. pleuropneumoniae* strains, encoding SM and SA resistance. These plasmids, pVM104 and pVM106, were different from pMS260 in size, but had 2.3 megadaltons (Mdal) in size.

Most of nonconjugative SM and SA resistance of clinical isolates belonging to various species of *Enterobacteriaceae* and *Pseudomonas* were encoded by Inc.Q plasmid, molecular size of which was 8.9 Kb [1]. pMS260 was different from one of the representative Inc.Q plasmids, Rlb679,

both in the molecular weight and in the number of cutting sites by restriction enzymes. And these plasmids, pMS260 and Rlb679 could coexist each other, indicating that pMS260 did not belong to Inc.Q. And further, pMS260 was compatible with any of other 14 incompatible groups.

We assume that pMS260 was transferred to *H. pleuropneumoniae* from external sources by mobilization with such a conjugal plasmid like RP₄ of broad host range. These results may anticipate the wide distribution of nonconjugative plasmids like pMS260 among various causative agents for infectious disease of the respiratory organs. Some SM and SA resistance plasmids like pMS260 have been isolated in other countries from domestic animals [2, 6, 35]. It is interesting for us to know homologous aspect between their plasmids and pMS260.

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要 約

Haemophilus (Actinobacillus) pleuropneumoniae の薬剤感受性および R-プラスミドの分離：石井宏志・中曽根由かり・重原 進・本間邦俊・荒木康久¹⁾・伊豫部志津子¹⁾・橋本 一¹⁾(群馬県中央食肉衛生検査所, ¹⁾群馬大学医学部微生物学教室)——豚の肺病巣から *Haemophilus pleuropneumoniae* 56株を分離し、それらの株について薬剤感受性試験を実施したところ 2 株の耐性菌を得た。このうち 1 株(KH-195)は、TC単剤性であったが、プラスミドの分離はできなかった。他の 1 株(KH-265)は、SM および SA の 2 剤耐性で、約 8.3 Kb のプラスミド(pMS260)を保有していた。この pMS260 の性状を精査するにあたり、広域宿主非伝達性(Inc.Q)SM-SA 耐性プラスミドの 1 つ Rlb679 を比較のために用いた。pMS260 は Rlb679 とは、制限酵素により異なる切断点を持ち、不和合性試験では、Inc.Q を含む 14 群のプラスミドすべてと和合性であり、Rlb679 とは性質が異なることが分った。また、*E. coli* 間において 11 種類の伝達性プラスミドのうち pMS260 を可動化させたものは、Inc.P の RP₄、Inc.X の R6K および Inc.M の R446b の 3 種類であった。このうち伝達頻度の高かった RP₄ と R446b を用いて、pMS260 の宿主域を調べる目的で、他の呼吸器系疾患の原因菌に pMS260 を可動化させたところ、RP₄ により、*P.aeruginosa*, *P. multocida*, *B. bronchiseptica*, および *H. pleuropneumoniae* に伝達可能なことが分った。